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Adenovirus-mediated Transfer of the Muscle Glycogen Phosphorylase Gene into Hepatocytes Confers Altered Regulation of Glycogen Metabolism*

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The muscle isozyme of glycogen phosphorylase is potently activated by the allosteric ligand AMP, whereas the liver isozyme is not. In this study we have investigated the metabolic impact of expression of muscle phosphorylase in liver cells. To this end, we constructed a replication-defective, recombinant adenovirus containing the muscle glycogen phosphorylase cDNA (termed AdCMV-MGP) and used this system to infect hepatocytes in culture. AMP-activatable glycogen phosphorylase activity was increased 46-fold 6 days after infection of primary liver cells with AdCMV-MGP. Despite large increases in phosphorylase activity, glycogen levels were only slightly reduced in AdCMV-MGP-infected liver cells compared to uninfected cells or cells infected with wild-type adenovirus. The lack of correlation of phosphorylase activity and glycogen content suggests that the liver cell environment can inhibit the muscle phosphorylase isozyme. This inhibition can be overcome, however, by addition of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which increases AMP levels by 30-fold and causes a much larger decrease in glycogen levels in AdCMV-MGP-infected cells than in uninfected or wild-type adenovirus-infected controls. CCCP treatment also caused a preferential decrease in glycogen content relative to glucagon treatment in AdCMV-MGP-infected hepatocytes (74% *versus* 11%, respectively), even though the two drugs caused equal increases in phosphorylase *a* activity. Introduction of muscle phosphorylase into hepatocytes therefore confers a capacity for glycogenolytic response to effectors that is not provided by the endogenous liver phosphorylase isozyme. The remarkable efficiency of adenovirus-mediated gene transfer into primary hepatocytes and the demonstration of altered regulation of glycogen metabolism as a consequence of expression of a non-cognate phosphorylase isozyme may have implications for gene therapy of glycogen storage diseases.

Glycogen is the storage form of the simple sugar glucose, and its presence in mammalian tissues provides a source of metabolic energy for periods of catabolic activity. Digestion of glycogen to yield glucose 1-phosphate (Glc-1-P)[1] is accomplished by the rate-limiting enzyme of glycogenolysis, glycogen phosphorylase. There are three distinct genes encoding glycogen phosphorylases in mammals, termed muscle, liver, and brain after the tissues in which they are preferentially expressed (1–3), that map to separate chromosomes in humans (4, 5) and mice (6). The proteins encoded by the three human glycogen phosphorylase genes are 80–83% identical in primary sequence (1–3, 7). Despite this high level of sequence conservation, the three phosphorylase isoforms have distinctive functional characteristics that are reflected in their different physiological roles. Thus, muscle phosphorylase is acutely regulated not only by phosphorylation (activation) and dephosphorylation (inactivation) at Ser-14, but is also sensitive to a number of allosteric effectors, including the activator AMP, and the inhibitors glucose, glucose 6-phosphate, ATP, and purine nucleotides (1, 8–10). Responsiveness to metabolic intermediates, especially AMP and related molecules, is in keeping with a physiological role for muscle phosphorylase of maintaining a high energy charge in an energy-requiring tissue. Liver phosphorylase, in contrast, is generally less sensitive to allosteric regulation than muscle phosphorylase. The liver enzyme is particularly unresponsive to activation by AMP, consistent with its primary role as a supplier of free glucose for extrahepatic metabolism, a function that might be compromised by inappropriate sensitivity to alterations in AMP levels.

Recently, we have begun to dissect the structural determinants of isozyme-specific allosteric regulation, with particular emphasis on the vastly different responses of the liver and muscle isozymes to AMP. This work has involved detailed *in vitro* kinetic studies on purified proteins (11). Such approaches do not, however, address the functional significance of allosteric regulation in intact cells. In this context, it is interesting to note that the phenotypic consequence of phosphorylase kinase deficiency is different in liver and muscle. Thus, the I-strain mouse, which specifically lacks muscle phosphorylase kinase, has only mildly elevated muscle glycogen levels and exhibits no obvious impairment when subjected to a regimen of intense exercise, suggesting that AMP activation of muscle phosphorylase compensates effectively for the lack of covalent activation (12, 13). The *gsd/gsd* rat, in contrast, lacks liver phosphorylase kinase and contains hugely elevated levels of hepatic glycogen, indicating that allosteric activation does not compensate for the phosphorylase kinase deficiency in this tissue (14). These data suggest that introduction of either muscle phophorylase or liver phosphorylase(s) engineered for AMP responsiveness into liver cells might impart altered modes of phosphorylase activation, leading to changes in the regulation of glycogen metabolism in response to physiological signals. In the current study, we have tested this idea by using recombinant adenovirus to transfer the muscle phosphorylase cDNA into liver cells. Our results confirm the hypothesis and show that the recombinant adenovirus system is an extremely efficient means of introducing metabolic regulatory proteins into non-proliferating cells for evaluation of their impact on metabolic flux. This approach may also have implications for studies involving transfer of genes encoding metabolic enzymes into cells and tissues of animals with glycogen storage diseases.

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[1] The abbreviations used are: Glc-1-P, glucose 1-phosphate; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; CMV, cytomegalovirus; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

MATERIALS AND METHODS

Cell Isolation and Culture—Hepatocytes were isolated from 24-h fasted adult male Wistar rats (180–225 g) by perfusion of the liver with a collagenase solution as described (15). Cells were plated onto collagen-coated plastic dishes (Corning) at a density of 6 × 10^4 cells/cm^2 in DMEM (4.5 g/liter glucose; GIBCO) supplemented with 10% fetal bovine serum, 100 nM insulin (Boehringer) and 100 nM dexamethasone (Sigma). After cell attachment (4–5 h), the medium was replaced with DMEM containing 0.2% bovine serum albumin (Sigma), 1 nM insulin, and 10 nM dexamethasone and was subsequently changed on a daily basis.

Preparation of Recombinant Adenovirus and Cell Infection—Recombinant adenovirus containing the cDNA encoding rabbit muscle glycogen phosphorylase (16) was prepared by the strategy outlined in Fig. 1. Briefly, the pACCMVpLpA vector was prepared by insertion of the constitutive cytomegalovirus (CMV) early gene promoter/enhancer, the pUC 18 polylinker, and a fragment of the SV40 genome that includes the small T-antigen intron and the polyadenylation signal, into the previously described pAC vector (17). A 2.56-kilobase pair *NdeI*/*Hin*dIII fragment of the rabbit muscle glycogen phosphorylase cDNA that includes all of the protein coding region was then inserted into the pACCMVpLpA vector. The resulting plasmid was cotransfected with pJM17 (18) into 293 cells (AdE1A-transformed human embryonic kidney cells; Ref. 19) by calcium phosphate/DNA coprecipitation. pJM17 encodes a full-length adenovirus 5 genome interrupted by the insertion of the bacterial plasmid pBRX at position 3.7 map units, thereby exceeding the packaging limit for adenovirus (18). Homologous recombination between the recombinant pACCMVpLpA plasmid and the pJM17 plasmid in 293 cells generates a genome of packageable size in which the adenovirus early region 1 is replaced by the cloned chimeric gene, rendering the recombinant virus replication defective. The resulting virus was named AdCMV-MGP. The presence of the phosphorylase insert in AdCMV-MGP viral DNA was confirmed by restriction enzyme digestion and Southern blotting using an oligonucleotide (5′-GCGTTGGGGTCTG-GCCTAGGGTATCTT-3′) complementary to the 3′ end of the rabbit muscle phosphorylase cDNA (data not shown). Appropriate viral plaques were expanded into stocks containing 5–50 × 10^7 plaque-forming units/ml and stored in DMEM supplemented with 10% fetal bovine serum. Hepatocytes were infected after cell attachment to plates by incubation with stocks of wild-type adenovirus type 5 or the recombinant AdCMV-MGP virus for 1 h at a multiplicity of infection of 5.

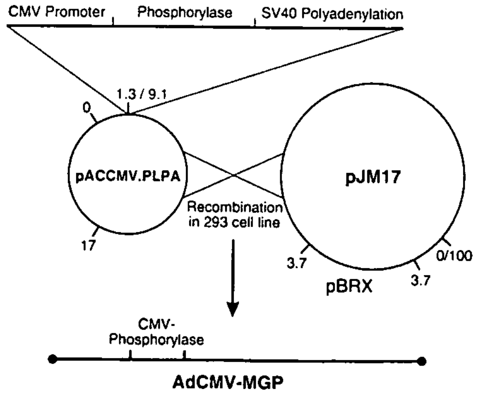

FIG. 1. **Schematic representation of the strategy used for construction of AdCMV-MGP recombinant adenovirus.** The recombinant virus contains a transcription unit consisting of the CMV promoter/enhancer, the rabbit muscle glycogen phosphorylase cDNA, and an SV40 polyadenylation cassette (see "Materials and Methods" for details).

In Situ mRNA Hybridization—The efficiency of infection with AdCMV-MGP was evaluated by performing *in situ* hybridization on AdCMV-MGP-infected and control cells. Cells were harvested 4 days after infection by light trypsinization, washed with phosphate-buffered saline (PBS) and spotted onto silane-coated microscope slides and allowed to settle. Immediately after air-drying, the slides were washed in PBS and treated with fixative solution A (4% paraformaldehyde, 100 mM sodium acetate, pH 6.5) for 10 min, and then with fixative solution B (4% paraformaldehyde, 0.05% glutaraldehyde, and 100 mM sodium tetraborate, pH 9.5) for 10 min. *In situ* hybridization was performed by preparing the rabbit muscle phosphorylase cDNA probe (20) in the presence of digoxigenin-11-dUTP (Boehringer Mannheim). Digoxigenin-labeled probe was then hybridized to cellular muscle phosphorylase mRNA using previously described hybridization and washing conditions (21). Hybridization events were visualized by treating the cells with anti-digoxigenin conjugated to alkaline phosphatase (dilution of 1:400) and subsequent incubation with blocking reagents and alkaline phosphate substrates as prescribed by the manufacturer (Boehringer Mannheim).

Assays of Phosphorylase Activity—Glycogen phosphorylase activities were assayed in hepatocyte extracts by native gel (11, 20) and radioisotopic (11, 22) techniques. For both methods, extracts were prepared by homogenization in a buffer consisting of 10 mM Tris-HCl, pH 7.0, 150 mM KF, 15 mM EDTA, 15 mM 2-mercaptoethanol, 10 μg/ml leupeptin, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. For gel assays, supernatant aliquots were electrophoresed on three identically prepared nondenaturing gels, soaked in buffers containing substrates and various activators, and stained to visualize newly formed glycogen, exactly as described (11, 20). In later gel assay experiments, cleared hepatocyte extracts were treated with purified rabbit muscle phosphorylase kinase as described (11, 23) using 48 μg of supernatant protein and 0.75 μg of enzyme per reaction. For assays monitoring the conversion of [U-^{14}C]glucose 1-phosphate (Glc-1-P) into glycogen (11, 22), the final assay mixture contained 75 mM Glc-1-P, 125 mM KF, and 0.6% glycogen, and labeled Glc-1-P at 0.04 μCi/assay. Glycogen phosphorylase *a* activity was determined by addition of 1 mM caffeine to the assay. In other experiments, AMP activation of phosphorylase *b* was measured by addition of the activator at a final concentration of 5 mM.

Measurements of Glycogen Metabolism—Primary hepatocytes (uninfected or 4 days after infection with wild-type adenovirus or AdCMV-MGP) were preincubated for 4 h in DMEM, 4.5 g/liter glucose, supplemented with 10 mM lactate, 1 mM pyruvate, and 100 nM insulin to maximize glycogen accumulation. Cell monolayers were rinsed with PBS, and subsequent incubations were carried out in DMEM lacking glucose. In addition to control experiments in which cells received no additions, the effects of 1 nM glucagon (Sigma) or 25 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Sigma) were studied. Glycogen phosphorylase *a* activity and glycogen content were measured after 20 min or 1 h of incubation, respectively.

Glycogen content was measured by scraping cells into 30% KOH, boiling the homogenate for 15 min, and centrifuging at 5,000 × *g* for 15 min. The cleared supernatants were spotted onto Whatman 31 ET paper, and glycogen was precipitated by immersing the papers in ice-cold 66% ethanol. After two additional washes in ethanol, the papers were air-dried and incubated with α-glucosidase (Sigma) as described (24). Glycogen-glucose was then measured enzymatically in a Cobas-Bio autoanalyzer with a Glucoquant (Boehringer Mannheim) kit.

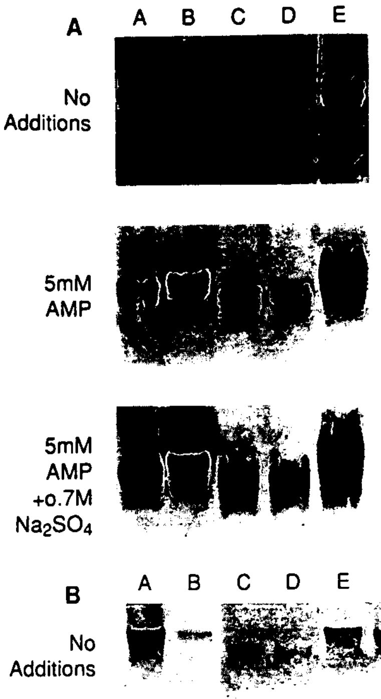

FIG. 2. **Activity gel assays of phosphorylase activities in AdCMV-MGP-infected and control hepatocytes.** *Panel A*, samples were electrophoresed on three identically prepared nondenaturing gels, which were then incubated in a buffer containing phosphorylase substrates and no further additions, 5 mM AMP, or 5 mM AMP + 0.7 M Na_2SO_4, as indicated to the *left* of each *panel*. *Lanes A* and *B* contain 0.15 μg of purified rabbit muscle glycogen phosphorylase *a* and *b*, respectively (Sigma). The following lanes contain extracts (160 μg of total protein/lane) from hepatocytes that were untreated (*lane C*), treated with wild-type adenovirus 5 (*lane D*), or treated with AdCMV-MGP (*lane E*). The migration position of phosphorylase activity in the gel is visualized by staining newly formed glycogen with an iodine solution (11, 20). The extracts in *panel A* were not treated with phosphorylase kinase. *Panel B*, *lanes A* and *B* contain 0.15 μg of purified rabbit muscle glycogen phosphorylase *a* and *b*, respectively. Samples in *lanes C–G* were treated with purified phosphorylase kinase as described under "Materials and Methods" prior to electrophoresis. These lanes contained the following: *lanes C–E*, 20 μg of total protein/lane from hepatocytes that were untreated (*lane C*), treated with wild-type adenovirus 5 (*lane D*), or treated with AdCMV-MGP (*lane E*); *lane F*, 0.15 μg of purified muscle phosphorylase; *lane G*, 0.9 μg of purified liver phosphorylase (11).

RESULTS

Efficiency of Expression Evaluated by in Situ Hybridization—The majority of AdCMV-MGP-infected hepatocytes that were hybridized with digoxigenin-labeled muscle phosphorylase cDNA and treated with anti-digoxigenin-alkaline phosphatase and substrates were clearly stained purple while uninfected cells or cells treated with wild-type adenovirus were uniformly translucent.[2] Counting of cells from 15 different photographed sections of slides revealed that 581 of a total of 675 (86.1%) AdCMV-MGP-infected cells were stained. In contrast, none of a total of 347 control cells (uninfected + wild-type virus-infected) were stained.

Phosphorylase Isoform Expression Assayed with a Native Gel System—Fig. 2*A* shows the results of a native gel activity assay using hepatocyte supernatants prepared in the presence of a phosphatase inhibitor (150 mM KF) and without phosphorylase kinase treatment. Control lanes contain commercially purified (Sigma) rabbit muscle phosphorylase *a* (*lane A*) or *b* (*lane B*). As expected (11, 20), muscle phosphorylase *a* is maximally active under all assay conditions studied, while muscle phosphorylase *b* is potently activated in the presence of AMP alone, approaching the maximal activity induced by incubation in the presence of 5 mM AMP and 0.7 M Na_2SO_4. The latter agent is a potent allosteric activator of all glycogen phosphorylase isoforms (11, 20, 25), and is used in this assay to express total phosphorylase activity. *Lanes C* and *D* contain supernatants prepared from uninfected hepatocytes and from hepatocytes 4 days after treatment with wild-type adenovirus, respectively. In the absence of any effectors it can be seen that the endogenous liver phosphorylase activity migrates as a doublet, probably consisting of the *a* and *b* forms of the enzyme. Incubation of the gel in the presence of 5 mM AMP slightly enhances activity, while strong activation requires the combination of AMP + Na_2SO_4, a result consistent with the relative lack of sensitivity of liver phosphorylase to AMP activation (reviewed in Ref. 1). The phosphorylase activity of primary hepatocytes has a greater mobility in the native gel assay than muscle phosphorylase, due to a distinct predicted isoelectric point (20). Four days after infection with the AdCMV-MGP virus, the migration position and intensity of the phosphorylase activity changes dramatically, as shown in *lane E*. The dominant band now comigrates with the muscle phosphorylase standards, and like purified muscle phosphorylase *b*, is completely activated in the presence of 5 mM AMP alone. These data demonstrate successful expression of the muscle phosphorylase isoform at high levels in primary hepatocytes via the adenovirus system.

To prove that the doublet of endogenous activity seen in Fig. 2*A* was in fact comprised of *a* and *b* forms of liver phosphorylase, we repeated these experiments after treatment of the cleared supernatants with purified phosphorylase kinase. As seen in Fig. 2*B*, kinasing of liver extracts that were untreated or treated with wild-type adenovirus, respectively (*lanes C* and D), resolved the doublet of activity seen in Fig. 2*A* into a single species, consistent with conversion of all endogenous liver phosphorylase activity to the *a* form. As shown in *lane E*, 4 days after treatment with AdCMV-MGP, the phosphorylase kinase-treated extract contains a single band of activity that comigrates with the muscle phosphorylase controls in *lanes A* and *B* and with Sigma muscle phosphorylase *b* that has been treated with phosphorylase kinase (*lane F*). *Lane G* contains native liver phosphorylase, expressed in bacteria and purified to homogeneity as previously described (11), and treated with phosphorylase kinase. The migration position of this activity is identical to that seen in extracts from untreated hepatocytes or hepatocytes treated with wild-type adenovirus (*lanes C* and *D*).

Phosphorylase Activity Measured by Radioisotopic Assay—The native gel activity assay demonstrates clearly a dramatic increase in expression of a phosphorylase activity that comigrates with purified muscle phosphorylase in AdCMV-MGP-infected cells. While these data are useful in defining the isoform that is responsible for changes in activity, they are not quantitative. To address this issue, we performed radioisotopic enzymatic assays (11, 21) of glycogen phosphorylase activities in hepatocyte extracts. Fig. 3 shows the time-dependent changes in phosphorylase activity observed in hepatocytes that were untreated (*panel A*), treated with wild-type adenovirus (*panel B*), or treated with AdCMV-MGP (*panel C*). Consistent with the data in Fig. 2, untreated hepatocytes or hepatocytes treated with wild-type adenovirus contained phosphorylase activity that was only slightly induced (10–33%) by the addition of 5 mM AMP to the assay. Infection with AdCMV-MGP induced an 8–10-fold increase in basal phosphorylase activity relative to the two control hepatocyte

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[2] Photographs of treated cells are available upon request.

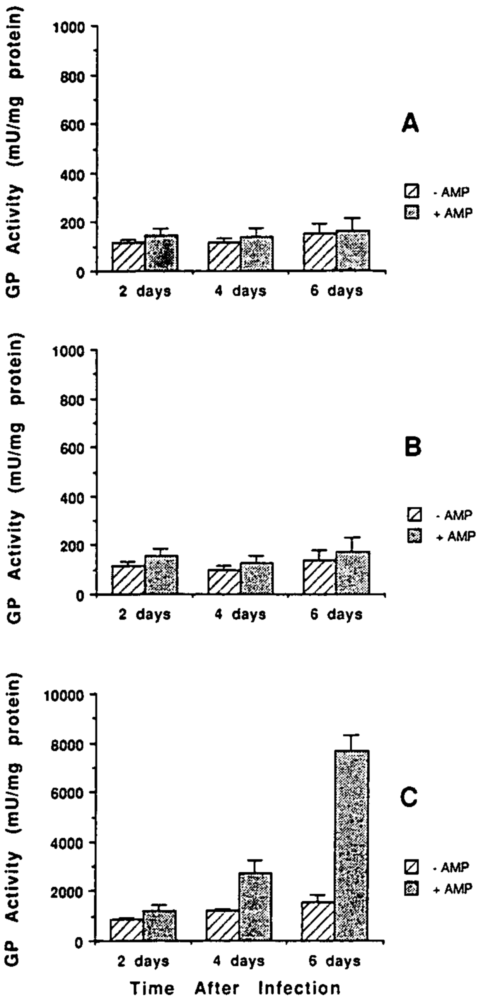

FIG. 3. **Time course of expression of AMP-activated phosphorylase activity.** Hepatocytes were isolated and cultured *in vitro* for the number of days indicated absent virus infection (*panel A*), or after infection with wild-type adenovirus 5 (*panel B*) or AdCMV-MGP (*panel C*). Glycogen phosphorylase activity was then measured in the presence and absence of 5 mM AMP (see *legends* to the *right* of each *panel*) as described under "Materials and Methods." Note the difference in scales when comparing *panel C* to *panels A* and *B*. Data are expressed as the mean ± S.E. for four independent experiments.

preparations (compare *−AMP bar* in *panel C* to *−AMP bar* in *panels A* and *B*). A time-dependent increase in AMP-activatable phophorylase activity was observed in cells infected with AdCMV-MGP. Six days after infection with AdCMV-MGP, phosphorylase in hepatocyte extracts was activated 5-fold by AMP (Fig. 3*C*) and total AMP-activated phosphorylase activity was 46-fold higher than in either the untreated or native virus-infected control cells.

Treatment of Cells with Glucagon and CCCP—The effect of overexpression of muscle glycogen phosphorylase on glycogen metabolism in primary hepatocytes was evaluated by administration of glucagon or CCCP (the latter is a metabolic inhibitor that lowers ATP and increases AMP levels; Ref. 26). The effects of these compounds on glycogen phosphorylase *a* activity (measured in the presence of caffeine to inhibit phosphorylase *b*) are shown in Table I. Infection of hepatocytes with AdCMV-MGP resulted in a 4.7-fold increase in basal phosphorylase *a* activity ($p < 0.001$ compared to uninfected or wild-type virus-infected cells). Glucagon and CCCP caused small but statistically significant increases in phosphorylase *a* activity, ranging from 24 to 100% over control values in the three hepatocyte preparations ($p \leq 0.01$ for all experiments).

TABLE I

Glycogen phosphorylase a activity in hepatocyte extracts

Cells were treated with AdCMV-MGP virus (containing muscle phosphorylase), wild-type adenovirus 5, or vehicle (uninfected) and cultured for 4 days. Glucagon (1 nM) or CCCP (25 μM) was then administered for 20 min and cells harvested for glycogen phosphorylase *a* assay in the presence of caffeine, an inhibitor of phosphorylase *b* (see "Materials and Methods" for details). Data are expressed as milliunits/mg protein and represent the mean ± S.E. of four independent experiments.

Hepatocyte treatment	No addition	Glucagon	CCCP
Uninfected	63 ± 1	94 ± 5[a]	84 ± 4[a]
Wild-type virus	63 ± 2	78 ± 3[a]	81 ± 4[a]
AdCMV-MGP	293 ± 36	515 ± 46[a]	594 ± 66[a]

[a] Statistically significant differences ($p \leq 0.01$) in phosphorylase *a* activity compared to control cells that received no addition. Within each experimental group of hepatocytes, comparison of glucagon *versus* CCCP treatment revealed no significant differences in phosphorylase *a* activities.

TABLE II

Glycogen content in hepatocyte extracts

Cells were treated with AdCMV-MGP, wild-type adenovirus 5, or vehicle (uninfected) and cultured for 4 days. Glucagon (1 nM) or CCCP (25 μM) were then administered for 1 h and cells harvested for assay of glycogen content as described under "Materials and Methods." Data are expressed as μg of glycogen/mg of protein and represent the mean ± S.E. for four independent experiments. Statistically significant changes in glycogen content are indicated by the symbols * ($p \leq 0.01$) and ** ($p < 0.001$) referring to the comparison of glycogen content in the presence of CCCP to glycogen content in the presence of glucagon.

Hepatocyte treatment	No addition	Glucagon	CCCP
Uninfected	388 ± 14	336 ± 15	231 ± 16*
Wild-type virus	412 ± 18	344 ± 16	243 ± 21*
AdCMV-MGP	338 ± 23	300 ± 14	89 ± 16**[a]

[a] $p < 0.001$, refers to the comparison of glycogen content in CCCP-treated AdCMV-MGP-infected cells to glycogen content in either group of CCCP-treated control cells.

The relatively modest effect of glucagon on phosphorylase *a* activity in these experiments is probably due to the preincubation of the cells with high concentrations of insulin (27). There was no significant difference in phosphorylase *a* activity in glucagon- *versus* CCCP-treated cells for any of the experimental groups.

The effect of glucagon and CCCP on glycogen content was measured in parallel hepatocyte cultures. As shown in Table II, infection of hepatocytes with AdCMV-MGP only modestly reduced basal glycogen content relative to uninfected or wild-type virus-infected cells. Treatment of uninfected or native-virus treated hepatocytes with glucagon caused decreases in glycogen content of 14 and 17% relative to basal levels, respectively ($p < 0.05$ for both groups), while the hormone lowered glycogen levels by 11% in cells infected with AdCMV-MGP (not significant). CCCP reduced glycogen content by 40 and 41% in uninfected and wild-type adenovirus-infected hepatocytes relative to cells that received no treatment ($p \leq 0.01$ for both comparisons). In AdCMV-MGP cells the CCCP effect was dramatically enhanced, with a decrease in glycogen content of 74% relative to AdCMV-MGP cells that received no drug; the glycogen levels in CCCP-treated, AdCMV-MGP-infected cells were significantly less than in CCCP-treated uninfected or wild-type virus-infected cells ($p < 0.001$). In addition, glycogen content was significantly less in CCCP-treated *versus* glucagon-treated AdCMV-MGP-infected cells ($p < 0.001$).

DISCUSSION

Much of our current understanding of isozyme diversity, allosteric regulation, and cAMP-mediated functional alteration of enzymes by covalent phosphorylation in mammalian systems originated in classical studies on the family of glycogen phosphorylase proteins (reviewed in Ref. 1). More recently, application of molecular biology (1–6, 11) and crystallography (8–10) to the phosphorylases has yielded a wealth of information about the structural basis for the regulation of enzyme activity. Despite these advances, a number of important questions remain unanswered. One such issue that we have been addressing by site-directed mutagenesis and domain substitution is the structural basis for the dramatic difference in response to AMP allosteric activation exhibited by the liver (poorly activated) and muscle (potently activated) phosphorylase *b* enzymes (11).

With the advent of new gene transfer technologies, a related issue can be addressed, namely the metabolic effect of transferring a tissue-specific isozyme of phosphorylase into a tissue in which it is not normally found. In the current work, we have specifically evaluated the effect of introducing the AMP-activatable muscle glycogen phosphorylase isozyme into primary hepatocytes, which normally express only the AMP-insensitive liver isozyme.

The muscle phosphorylase cDNA was expressed with excellent efficiency in primary hepatocytes using the adenovirus vector/CMV promoter system. *In situ* hybridization experiments indicate that 86% of cells infected with AdCMV-MGP express the muscle phosphorylase mRNA, in keeping with previous reports of high efficiency adenovirus-mediated expression in other cell systems (28). Expression of the transgene was easily detectable 48 h after infection with the AdCMV-MGP virus, and levels of expression increased throughout the 6-day experimental period, with a maximal increase in activity of 46-fold achieved on the 6th day after infection. The possibility that the increased phosphorylase activity is caused by viral effects on endogenous phosphorylase expression is ruled out by two observations: 1) infection with wild-type adenovirus 5 causes no enhancement of phosphorylase activity, and 2) gel activity measurements indicate that the dominant phosphorylase band in extracts from rat hepatocytes infected with AdCMV-MGP comigrates with the rabbit muscle phosphorylase control activities.

The most striking consequence of muscle phosphorylase overexpression in hepatocytes is a strongly enhanced glycogenolytic response to the metabolic inhibitor CCCP. The enhancement is clearly evident when comparing AdCMV-MGP-infected cells to uninfected or native virus-infected controls. It is also obvious when comparing glucagon-treated *versus* CCCP-treated AdCMV-MGP-infected hepatocytes. The most plausible interpretation of the data is that the potent CCCP effect on AdCMV-MGP-infected cells is largely due to AMP allosteric activation of the introduced muscle phosphorylase. In support of this construct, CCCP has been shown to cause an increase in AMP from a basal (untreated) level of 0.1 μmol/g liver to 3 μmol/g liver after only 10 min of incubation (26). A level of 3 μmol/g AMP is roughly equivalent to a concentration of 5 mM, an amount that strongly activates muscle phosphorylase *b in vitro* (29). Furthermore, the level of phosphorylase *a* activity was similar in glucagon- and CCCP-treated hepatocytes, meaning that covalent activation cannot explain the greatly enhanced glycogenolytic effect of CCCP relative to glucagon. The increase in phosphorylase *a* activity in all groups of CCCP-treated hepatocytes is consistent with previous reports (26) and may be due to activation of phosphorylase kinase by Ca^{2+} or nucleotides (30).

An intriguing finding of this study is that increasing phosphorylase *a* activity many-fold by introduction of the muscle phosphorylase isozyme into hepatocytes has little effect on glycogen degradation, except in the presence of CCCP (see Tables I and II). Possible interpretations include the following: 1) intact hepatocytes lack a ligand or factor that is present in muscle tissue and that is required for full expression of the enzymatic activity, and 2) the concentrations of certain allosteric inhibitors are higher in hepatocytes than in muscle cells. Candidate inhibitory ligands could include glucose and UDPglucose. Muscle phosphorylase is inhibited by UDPglucose with a K_i of 0.9 mM (31). The concentration of this ligand in muscle is only 40 μM, but in liver, levels of UDPglucose that could become relevant for inhibition of phosphorylase (0.5 mM) have been reported (32). A suppressor role for the allosteric inhibitor glucose is suggested by the fact that muscle contains the insulin-requiring glucose transporter GLUT-4, while liver contains GLUT-2, which has a high capacity for transport and no insulin requirement. As a consequence of this expression pattern and relative rates of glucose usage in the two tissues, the intracellular concentration of glucose in hepatocytes is usually similar to the circulating level, while free glucose is difficult to detect inside muscle cells. Large increases in AMP concentration, like those induced by CCCP administration, are apparently sufficient to overcome the lack of activating factors or effects of inhibitors. It should be noted that increases in the levels of the phosphorylase substrate P_i could also contribute, since such increases are thought to enhance phosphorylase *a* activity in hepatocytes subjected to anoxia or treated with KCN (33). It will be of interest to determine whether introduction of native liver phosphorylase or liver phosphorylase proteins engineered for AMP activation (11) into liver cells allows greater glycogen degradation under basal conditions or upon glucagon stimulation than observed in the face of muscle phosphorylase overexpression.

Replication-defective adenovirus represents an increasingly popular strategy for gene transfer (28, 34–37). Compared to more commonly used retroviral systems, adenovirus-mediated gene transfer may have several distinct advantages in that expression of foreign genes is not restricted to replicating cells, large DNA inserts are accommodated, and surprisingly persistent expression is maintained (34, 35) despite the fact that the vector does not integrate efficiently into the host cell genome (38). A currently emphasized gene therapy strategy involves the isolation of primary tissue (liver or hematopoetic stem cells, for example), infection of the cells *in vitro* with recombinant retroviruses, and reimplantation of the engineered cells (39, 40). Familial hypercholesterolemia and phenylketonuria are examples of metabolic diseases involving hepatic lesions that may be amenable to this therapeutic approach, since even small systemic increases in the deficient protein (the LDL receptor or phenylalanine hydroxylase, respectively) should be sufficient to cause major improvements in the clinical phenotype (39–41). This approach will not work well for the glycogen storage diseases, however, since the deleterious effects of such syndromes are caused by the overproduction of a cellular intermediate (glycogen), as opposed to the overproduction of a metabolite in the circulation. Therapy for such disorders will likely require a method for efficient introduction of genes into cells in intact organs, even those in which cell division occurs at a low rate. The adenovirus system may be ideally suited for such *in situ* gene transfer strategies, as indicated by the data described herein, as well as recent experiments by others in which long term expression of introduced genes has been achieved by introduction of recombinant adenoviruses into whole animals (34, 35). The intense effort devoted to understanding the safety and efficacy of the retroviral vectors in recent years must now be duplicated for the recombinant adenovirus system.

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